UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

009262

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OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT **DIETHANOLAMINE SALT of 2,4-D:** Review of Mutagenicity and Teratology Studies.

FROM: Jess Rowland, M.S, Toxicologist Jess Rowland 2/10/92
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THRU: K.Clark Swentzel, Section Head K. Clark Swentzel 2/10/92
Section II, Toxicology Branch II
Health Effects Division (H7509C)
and
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STUDY IDENTIFICATIONS: **Submission: S401519 ID #:030016**

HED Project No. 1-2159 **Caswell No. 315 K**

MRID No(s): 419209-07 thru 419209-10 [Mutagenicity studies]
419866-02 [Range-finding Teratology study]
419209-06 [Teratology study]

Registrant: PBI Gordon Corporation, Kansas City, MO

ACTION REQUESTED: In-depth review of four mutagenicity studies, a range finding teratology study in rats, and a teratology study in rats with the diethanolamine salt of 2,4-dichlorophenoxyacetic acid.

RESPONSE: A separate Data Evaluation Report [DER] for each of the above referenced studies is attached. The results of each study are tabulated below:

STUDY	MRID No.	RESULTS	CORE CLASSIFICATION
Acute Oral-Mice	419209-07	LD_{50} = 862 mg/kg	Minimum
In vivo Micronucleus Assay	419209-08	Mice were given single oral doses of 60, 200 or 600 mg/kg. Non clastogenic; no significant increase in the frequency of MPE's. Toxic signs at the high dose: ataxia, languidness and squinted eyes	Acceptable
In vitro UDS Assay	419209-09	Concentration tested: 10 to 500 µg/mL. Negative; no appreciable increase in net nuclear grain counts of treated hepatocytes.	Acceptable
Ames Assay	419209-10	Concentration tested: 500 to 14,000 µg/plate. Non mutagenic with or without activation.	Acceptable
Range-Finding Rat Teratology	419866-02	Doses levels:0, 37, 75,150, or 300 mg/kg/day. Maternal and developmental toxicity at 150 & 300 mg/kg. Dose levels selected: 0, 15, 75, or 150 mg/kg	Not applicable
Teratology	419209-06	Dose levels: 0, 15, 75 or 150 mg/kg during days 6-15 of gestation. No maternal or developmental toxicity at 15. Maternal toxicity at 75 and 150 were decreases in body weight gain and food consumption. Developmental toxicity characterized by reduced fetal body weight [150 mg/kg/day] and fetal anomalies [75 and 150 mg/kg/day]. Maternal & Developmental NOEL = 15 mg/kg/day Maternal & Developmental LOEL = 75 mg/kg/day	Minimum

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SECONDARY REVIEWER: Byron T. Backus, Ph.D, Toxicologist
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DATA EVALUATION REPORT

1. DOSE-SELECTION STUDY IN MICE

STUDY TYPE: Acute Oral Toxicity **GUIDELINE: NA**

Caswell No. 315 K **MRID No. 419209-07** **HED PROJECT No. 1-2159**

TEST MATERIAL: Diethanolamine salt of 2,4-Dichlorophenoxyacetic acid

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Hazleton Laboratories America Inc, MD.

STUDY IDENTIFICATION: HLA 12216-0-459-PO

TITLE OF REPORT: Single Acute Exposure Dose Selection Study on Diethanolamine Salt of 2,4-Dichlorophenoxyacetic acid.

AUTHOR: James L. Ivett, Ph.D

REPORT DATE: June 20, 1990

SUMMARY: A dose-selection study was conducted with 2,4-D DEA in male and female ICR mice to select dose levels for an in vivo micronucleus assay. Groups of three male and three female mice received a single oral administration of 2,4-D DEA [73.8%] suspended in sterile deionized water at 400, 800, 1000, 1200 or 1400 mg/kg. Mice were sacrificed three days later. The oral LD_{50} was 862 mg/kg or 636.2 mg/kg 2,4-D DEA.

CORE CLASSIFICATION: Not applicable; this study is not a Guideline requirement.

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DATA EVALUATION REPORT

2. IN VIVO MICRONUCLEUS ASSAY IN MICE

STUDY TYPE: Structural Chromosomal Aberrations **GUIDELINE:** 84-2(b)

Caswell No. 315 K MRID No. 419209-08 HED PROJECT No. 1-2159

TEST MATERIAL: Diethanolamine salt of 2,4-D Acid

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Hazleton Laboratories America Inc, MD.

STUDY IDENTIFICATION: HLA 12216-0-455

TITLE OF REPORT: Mutagenicity Test on Diethanolamine Salt of 2,4-Dichlorophenoxyacetic Acid In vivo Micronucleus Assay.

AUTHOR: James L. Ivett, Ph.D

REPORT DATE: December 4, 1990

SUMMARY: The ability of the test material, Diethanolamine Salt of 2,4-Dichlorophenoxyacetic Acid [73.8%], to induce micronuclei in bone marrow polychromatic erythrocytes of mice was tested in an in vivo micronucleus assay. Groups of five male and five female ICR mice were given a single oral gavage administration of the test material at doses of 60, 200, or 600 mg/kg [equivalent to 44.28, 147.6 or 442.8 mg/kg 2,4-D DEA] and were sacrificed 24, 48 and 72 hours after dosing for extraction of the bone marrow. Vehicle and positive control groups euthanatized 24 hours after dosing were included in the assay. 2,4-D DEA did not induce a significant increase in the frequency of micronucleated polychromatic erythrocytes [PCEs] harvested at 24, 48 and 72 hours posttreatment. Toxic signs which included ataxia, languidness and squinted eyes were seen in male mice at the high dose. Under the conditions of this assay, the diethanolamine salt of 2,4-D is considered negative in the mouse bone marrow micronucleus test.

V. CORE CLASSSIFICATION

Acceptable; this study satisfies the Guideline requirement [84-2(b)] for genetic effects Category II, Structural Chromosomal Aberrations.

I. INTRODUCTION

This Data Evaluation Report summarizes the experimental procedures and the results of an in vivo micronucleus assay in mice.

II. MATERIALS and METHODS

1. Test Material:

Name: 2,4-D diethanolamine [2,4-D DEA]
Description: Amber liquid
Batch No.: 0017
Purity: 73.8%
Acid Equivalent: 50%
Vehicle: Sterile deionized water
Analysis: Concentration and stability [24-hr] analysis

2. Positive/ Negative Controls:

Positive Control: Cyclophosphamide [CP, Sigma]
Lot No.: 67F-0155
Vehicle Control: Sterile deionized water
Lot No.: Lot G

3. Test Animals:

Species: Mice
Strain: ICR
Sex: Male and Female
Weight: 30.6 - 39.8 g, M; 21.3 - 28.7 g, F
Housing: 5/cage
Food: Purina Certified Chow #5002
Water: tap water ad libitum
Environment: Temperature-72 ± 6° F; Humidity-50 ± 20%; Light- 12 hr photo cycle.

4. Treatment:

Groups of five male and five female mice were given a single oral administration of the test material in sterile deionized water at 60, 200 or 600 mg/kg. The dose selection was based on the results of a dose selection study [HLA 12216-0-459; MRID No. 419209-07]. In a secondary dose group, 5 mice/sex was dosed with 600 mg/kg; animals in this group were used only to replace animals that died in the primary group. The positive control group consisting of 5 male and 5 female mice received a single oral dose of Cyclophosphamide at 80 mg/kg and the vehicle control group [5/sex] received sterile deionized water at a volume of 10 ml/kg.

5. Bone marrow harvest, slide preparation and analysis:

At 24, 48, and 72 hours after treatment, the appropriate groups of animals were sacrificed by CO_2 asphyxiation. Sacrifice time for negative and positive control groups was 24 hours. Bone marrow cells were flushed from both tibiae, transferred to tubes containing fetal calf serum, centrifuged, resuspended, spread onto slides, and air dried. Prepared slides were fixed in methanol, stained in May-Gruenwald and Giemsa solutions, coverslipped, and coded. The coded slides were then scored for micronuclei in PCEs and the PCEs to normochromatic (NCE) cell ratio. The number of PCEs examined per animal was 1000 and the number of NCE examined was determined while scoring 1000 PCEs per animal. The normal frequency of micronuclei in this mouse strain is about 0.0 - 0.4%

6. Quality assurance measures:

A quality assurance statement was signed and dated 12/05/90. This date conforms to the review of the study phases and the draft and the final reports.

III. RESULTS

1. Analytical Determinations:

Data presented from the analytical determination of dosing solutions used indicated that the actual concentrations were within 10% of the target concentrations. Similarly, the test material was stable in sterile deionized water for at least 24 hours at room temperature under normal laboratory light.

2. Animal Observations:

Mice were observed for toxic signs and/or mortality immediately after dosing and periodically throughout the course of the study. All animals appeared normal and healthy immediately after dosing. Approximately 1-hour after dosing, all mice at 600 mg/kg were ataxic. Just prior to the 24 hour harvest, one male mice each from the high-dose group and the secondary dose group appeared languid with squinted eyes. All remaining animals appeared normal and remained healthy until the appropriate harvest times.

3. Micronucleus Assay:

As shown in Table 1, the three evaluated doses of 2,4-D DEA [60, 200, and 600 mg/kg] did not induce a significant increase in the frequency of MPEs in cells harvested from either male or female mice sacrificed at 24, 48, or 72 hours post treatment. The significant ($p < 0.05$) increase in micronuclei induction elicited by the positive control [80 mg CP] demonstrated the sensitivity of the assay to detect a clastogenic response.

IV. CONCLUSION

Under the conditions of this assay, the diethanolamine salt of 2,4-D did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes and is considered negative in the mouse bone marrow micronucleus test. There were no biologically significant differences between groups with respect to PCE:NCE ratios that could be related to exposure to the test material.

V. CORE CLASSSIFICATION

Acceptable; this study satisfies the Guideline requirement [84-2(b)] for genetic effects Category II, Structural Chromosomal Aberrations.

TABLE 1. Representative Results of the Micronucleus Assay in Mice with the Diethanolamine Salt of 2,4-D.

Treatment	Dose	Harvest Time (HR)	% MPEs Mean of 1000/mice ± S.E		Ratio PCE:NCE Mean ± S.E	
			Male	Female	Male	Female
Vehicle Control	10 ml/kg	24	0.04 ± 0.04	0.04 ± 0.02	0.43 ± 0.05	0.95 ± 0.18
Positive Control [CP]	80 mg/kg	24	1.84 ± 0.27*	1.94 ± 0.53*	0.44 ± 0.06	1.06 ± 0.11
2,4-D DEA	60 mg/kg	24	0.06 ± 0.02	0.10 ± 0.06	0.30 ± 0.04	0.63 ± 0.08
		48	0.00 ± 0.00	0.06 ± 0.04	0.46 ± 0.14	0.62 ± 0.06
		72	0.08 ± 0.05	0.06 ± 0.02	0.36 ± 0.05	0.64 ± 0.11
2,4-D DEA	200 mg/kg	24	0.08 ± 0.04	0.10 ± 0.03	0.25 ± 0.05	1.12 ± 0.13
		48	0.02 ± 0.02	0.06 ± 0.04	0.29 ± 0.04	0.60 ± 0.12
		72	0.08 ± 0.04	0.00 ± 0.00	0.35 ± 0.08	0.86 ± 0.13
2,4-D DEA	600 mg/kg	24	0.02 ± 0.02	0.12 ± 0.04	0.35 ± 0.05	0.66 ± 0.04
		48	0.02 ± 0.02	0.08 ± 0.05	0.30 ± 0.04	0.46 ± 0.07
		72	0.04 ± 0.02	0.00 ± 0.00	0.29 ± 0.05	0.63 ± 0.06

* Significantly greater than the corresponding vehichle control at $p < 0.05$
PCE= Polychromatic erythrocytes
NCE= Normochromatic erythrocytes

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DATA EVALUATION REPORT

3. UNSCHEDULED DNA SYNTHESIS ASSAY

STUDY TYPE: Other Genotoxic Effects **GUIDELINE:** 84-4

Caswell No. 315 K MRID No. 419209-09 HED PROJECT No. 1-2159

TEST MATERIAL: Diethanolamine salt of 2,4-D Acid

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Hazleton Laboratories America Inc, MD.

STUDY IDENTIFICATION: HLA 12216-0-447

TITLE OF REPORT: Mutagenicity Test on Diethanolamine Salt of 2,4-Dichlorophenoxyacetic Acid in the In vitro Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay.

AUTHOR: Marie E. McKeon, M.Phil.

REPORT DATE: December 20, 1990

SUMMARY: The test material, the diethanolamine salt of 2,4-D [73.8%], was tested in a Primary Rat Hepatocyte UDS Assay. In the initial testing, freshly prepared rat hepatocytes were exposed to the test material at concentrations ranging from 0.500 to 5000 µg/mL [equivalent to 0.369 to 3690 µg/mL 2,4-D DEA). Cytotoxicity was clearly demonstrated at concentrations ≥ 100 µg/mL. Based on these preliminary results, concentrations ranging from 10 to 500 µg/mL test material [equivalent to 7.38 to 369 µg/mL 2,4-D DEA] were selected for the UDS assay. None of the dose levels caused significant increases in the net nuclear grain counts of treated rat hepatocytes.

Under the conditons of this assay, there was no indication that exposure to the diethanolamine salt of 2,4-D at concentrations of up to 369 µg/mL (actual concentration, a.i) resulted in induction of UDS in rat hepatocytes.

V. CORE CLASSSIFICATION

Acceptable; this study satisfies the Guideline requirement [84-4] for genotoxic effects category III, Other Genotoxic Effects.

I. INTRODUCTION

This Data Evaluation Report summarizes the experimental procedures and the results of an in vitro rat primary hepatocyte UDS assay.

II. MATERIALS

1. Test Material:

Name: 2,4-D diethanolamine [2,4-D DEA]
Description: Amber liquid
Batch No.: 0017
Purity: 73.8%
Acid Equivalent: 50%
Analysis: Concentration and stability [24-hr] analysis

2. Indicator Cells:

Primary rat hepatocytes were obtained by the in situ perfusion of the liver of an adult male Fischer 344 rat weighing 237.5g purchased from Harlan Sprague Dawley, Inc.

3. Positive and Negative Controls:

Sterile distilled water at a final concentration of 10% was used as the solvent control, and 2-acetylaminofluorene (2-AAF) at 0.10 µg/mL was used as the postive control.

4. Medium:

WMEI: Williams Medium E supplemented with 2 mM L-glutamine and antibiotics; WME+: As above with 10% fetal calf serum.

III. METHODS

1. Cell Preparations:

a. Perfusion technique: The liver was perfused with Hank's balanced salts containing 0.5 mM EGTA and HEPES buffer, pH 7.2, for 4 minutes and with WMEI containing 50 to 100 units/mL collagenase for 10 minutes. The liver was excised, removed to a culture dish containing WMEI and collagenase, and mechanically dispersed to release the hepatocytes.

b. Hepatocyte harvest/culture preparations: Recovered cells were centrifuged, resuspended in WME+, counted and aliquoted (0.5×10^6 cells/3 mL WME+) onto plastic coverslips in a series of culture dishes. The cultures were placed in a humidified, 37° C, 5% CO_2 incubator for an approximately 2-hour attachment period. Unattached cells were removed; viable cells were refed and established as monolayer cultures.

2. Dose Selection:

Initially, 15 concentrations of the test material (0.5 to 5000 µg/mL) were assayed. When the viability estimate was obtained (21.4 hours after initiation of treatment), at least six of these doses were chosen for analysis of nuclear labeling, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies and proceeding to sucessively lower doses.

3. UDS Assay:

a. Dose levels: Test Material: 10, 25, 50, 100, 250, or 500 µg/mL [equivalent to 7.38, 18.45, 36.9, 73.8, 184.5 or 369 µg/mL 2,4-D DEA. Positive control: 2-AAF; 0.10 µg/mL

b. Treatment: Five replicate monolayer cultures were exposed to the six doses of the test material, negative control, or positive control for 18.6 hours in WMEI containing 10 µCi/mL [^{3}H] thymidine. Treated monolayers were washed twice with WMEI; two of the five replicates for each treatment group were used to determine cytotoxicity. These cultures were refed, reincubated, and monitored for cytotoxicity at 21.4 hours post treatment by trypan blue exclusion.

c. UDS slide preparation: The remaining three cultures were washed with media containing 1 mM thymidine. Treated hepatocytes, attached to coverslips, were exposed to 1% sodium citrate for approximately 10 minutes, fixed in acetic acid:ethanol (1:3), dried, and mounted.

d. Preparation of autoradiographs/grain development: slides were coated with Kodak NTB2 emulsion, dried for 7 days at 4°C in light-tight desiccant-containing boxes, developed in Kodak D-19, fixed, stained with Williams modified hematoxylin and eosin, coded, and counted.

e. Grain counting: The nuclear grains of morphologically normal cells (50/coverslip) for each test dose and negative and positive controls were counted microscopically. Net nuclear grain counts were determined by subtracting the average cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus from the nuclear grain count of each cell.

4. Evaluation Criteria:

a. Assay validity: For the assay to be considered valid, the following criteria must be satisfied: [1] hepatocytes recovered from the perfusion step and monolayer cultures used for the assay must show ≥ 70% viability; [2] the negative or solvent control should have net nuclear grain counts of -5.0 to 1.0, and ≤10% of the cells should contain ≥ 5 net nuclear grains/nucleus; [3] the positive control must demonstrate the sensitivity of the test system to detect UDS; [4] data must be obtained from at least two replicate cultures/and at least 50 cells per culture; and [5] the highest dose must show cytotoxicity, the limit of solubility, or reach the maximum recommended dose for this assay [5 mg/mL].

b. Positive response: The assay was considered positive if: [1] the increase in the mean net nuclear grain count was ≥5 grains/nucleus over the negative control value, and/or [2] the percentage of nuclei with ≥5 grains exceeeded 10% of the percentage observed in the negative control population.

5. Quality assurance measures:

A quality assurance statement was signed and dated 12/20/90. This date conforms to the review of the study phases and the draft and the final reports.

III. RESULTS

1. Analytical Determinations:

Data presented from the analytical determination of the lowest and highest dosing solutions used indicated that the actual concentrations were within 10% of the target concentrations. Similarly, the test material was found to be stable in sterile deionized water for at least 24 hours at room temperature.

2. UDS Assay:

In the initial trial consisting of 15 doses ranging from 0.500 to 5000 µg/mL, the test material was highly toxic at and above 1000 µg/mL and moderately toxic at 500 µg/mL (61.7% survival) and 250 µg/mL (76.6% survival). Dose levels at and below 100 µg/mL were non-toxic. Based on these results, the six dose levels (10 to 500 µg/mL) were selected for the main assay.

In the main assay, net nuclear grain counts at the scored levels were not significantly increased in the treated groups compared to the solvent control. Furthermore, no dose-related trend was evident. Representative results are presented in Table 2.

The minimum criteria for UDS in this assay were 1) a mean net nuclear grain count exceeding 3.39 and/or at least 15.3% of the nuclei containing 5 or more net nuclear grains. Both criteria were met by the positive control (mean net nuclear grain count:34.77; 10%% of cells with ≥5 MNNG).

V. CONCLUSION

Under the conditons of this assay, the test material containing 73.8% diethanolamine salt of 2,4-D did not induce a significant increase in the nuclear labelling of rat primary hepatocytes for an applied concentration range of 10 to 500 µg/mL [equivalent to 7.38 to 369 µg/mL 2,4-D DEA]. 2,4-D Diethanolamine salt was, therefore, evaluated as inactive in the in vitro Rat Primary Heaptocyte Assay.

V. CORE CLASSSIFICATION

Acceptable; this study satisfies the Guideline requirement [84-4] for genotoxic effects category III, Other Genotoxic Effects.

TABLE 2. Results of an UDS Assay in Primary Rat Hepatocytes with Diethanolamine Salt of 2,4-D.

Material	Concentration	Mean Net Nuclear Grains (NNG)[1]	% Cells with ≥5 Mean NNG[2]	Mean Cyto. Grains[3]	% Survival at 18.6 hours[4]
Solvent Control	10%	-1.61	5.33	17.41	100.0
Positive Control	0.10 µg/mL	34.77	100.00	18.77	91.0
2,4-D DEA [73.8%]*	500 µg/mL	1.05	9.33	9.01	61.7
	250 µg/mL	-2.64	1.33	13.63	76.6
	100 µg/mL	0.47	10.67	22.50	96.6
	50 µg/mL	0.32	14.00	15.64	96.6
	25 µg/mL	-1.23	2.00	14.80	99.6
	10 µg/mL	-2.33	2.00	15.80	ND

1= Average of net nuclear grain counts on triplicate coverslips (150 total cells). Net nuclear grains = Nuclear grain count - Average cytoplasmic grain count.

2= Average percentage of cells with greater than or equal to 5 net nuclear grains determined from triplicate coverslips (150 total cells).

3= Average of cytoplasmic grain counts determined from triplicate coverslips (150 total cells).

4= Survival = Number of viable cells per unit area relative to the solvent control.

*= Equivalent to 369, 184.5, 73.8, 36.9, 18.45 or 7.38 µg/mL 2,4-D DEA.

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SECONDARY REVIEWER: Byron T. Backus, Ph.D, Toxicologist
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DATA EVALUATION REPORT

4. GENE MUTATION ASSAY

STUDY TYPE: Gene Mutation **GUIDELINE:** 84-2(a)

Caswell No. 315 K **MRID No. 419209-010** **HED PROJECT No. 1-2159**

TEST MATERIAL: Diethanolamine salt of 2,4-D Acid

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Hazleton Laboratories America Inc, MD.

STUDY IDENTIFICATION: HLA 12216-0-401

TITLE OF REPORT: Mutagenicity Test on Diethanolamine Salt of 2,4-Dichlorophenoxyacetic Acid in the Salmonella/Mammalian Microsome Reverse Mutation Assay.

AUTHORS: Timothy E. Lawlor and Patricia A. Holloway

REPORT DATE: December 3, 1990

SUMMARY: The test material, containing 73.8% of the diethanolamine salt of 2,4-D, was tested in the Salmonella/Mammalian Microsome Reverse Mutation Assay. Salmonella typhimurium stains TA98, TA100, TA1535, TA1537 an TA1538 were exposed to test material at concentrations of 0, 500, 2500, 5000, 10,000 or 14,000 µg/plate [equivalent to 0, 369, 738, 1845, 3690, 7380, or 10332 µg/plate 2,4-D DEA] both in the presence and absence of microsomal enzymes prepared from Aroclor-induced rat liver [S9]. The test material did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of metabolic activation.

There was no evidence that the diethanolamine salt of 2,4-D was mutagenic under the conditions of this assay.

CORE CLASSSIFICATION

Acceptable: this study satisfies the Guideline requirement [84-2(a)] for genotoxic effects category I, Gene Mutation Test.

I. INTRODUCTION

This Data Evaluation Report summarizes the experimental procedures and the results of a Salmonella/microsomal assay.

II. MATERIALS

1. Test Material:

Name: 2,4-D diethanolamine [2,4-D DEA]
Description: Amber liquid
Batch No.: 0017
Purity: 73.8%
Acid Equivalent: 50%
Analysis: Concentration and stability [24-hr] analysis

2. Control Materials:

Negative Control: Distilled deionized water
Positive Control:

Nonactivation: Sodium azide 2.0 µg/plate TA100, TA1535
2-Nitrofluorene 1.0 µg/plate TA98, TA1538
ICR-191 2.0 µg/plate TA1537

Activation: 2-Aminoanthracene 2.5 µg/plate for all strains.

3. Metabolic Activation: The Aroclor 1252 induced rat liver homogenate was purchased commercially. The S9 mix was prepared immediately before its use in the assay. One mL of the microsomal enzyme reaction mixture (S9 mix) contained the following components:

H_2O	0.70 mL
1.00 M NaH_2PO_4/K_2HPO_4 (pH 7.4)	0.10 mL
0.25 M Glucose-6-phosphate	0.02 mL
0.10 M NAPD	0.04 mL
0.20 M $MgCl_2$/0.825 KCl	0.04 mL
S9	0.10 mL
	1.00 mL

4. Tester Strains Used:

S.typhimurium strains TA98, TA100, TA1535, TA1537, TA1538

III. METHODS

1. Concentrations Tested:

a. Preliminary Cytotoxicity Assay: In the dose-rangefinding study, ten doses (14, 150, 100, 140, 500, 1000, 1400, 5000, 10,000, and 14,000 µg/plate) were evaluated with or without metabolic activation in s. typhimurium strain TA100. Single plates were used per dose per condition.

b. Mutagenicity Assay: Six doses of test material (500, 1000, 2500, 5000, 10,000, and 14,000 µg/plate; equivalent to 369, 738, 1845, 3690, 7380, and 10332 µg/plate 2,4-D DEA) were evaluated with and without metabolic activation in all tester strains.

2. Protocol:

a. Plating Procedures: In general, similar procedures were used for the preliminary cytotoxicity and mutation assays.

To tubes containing 2.5 mL volumes of molten top agar, 100 µL of an overnight broth culture of the appropriate tester strain and 50 µL of the appropriate test material dose, vehicle, or positive control were added. For the S-9 activated test, 0.5 mL of the S9 cofactor mix was added to tubes containing 2.0 mL of top agar; tester strains and test and control solutions were added as described. The contents of the tubes were mixed, poured over Vogel-Bonner minimal medium E, and incubated at 37°C for 48 ± 8 hours. At the end of incubation, plates were either immediately scored for revertant colonies or were refrigerated and subsequently counted with an automatic colony counter. Means and standard deviations were determined for the mutation assay.

b. Sterility Controls; A sterility test was performed on the highest dose of the test material, and 0.5 mL of the S9 mix.

c. Evaluation Criteria;

i. Assay Validity: The assay was considered valid if the folllowing criteria were met: (1) the presence of the appropriate genetic markers was verified for each strain; (2) the spontaneous revertants of each strain fell within the reporting laboratory's acceptable ranges; (3) cell densities were $\geq 5.0 \times 10^8$ cells/mL; and (4) all positive controls caused at least a 3-fold increase in revertants per plate compared with the respective solvent control.

ii. Positive Response; The test material was considered positive if it caused a ≥ 2-fold increase in mean revertant colonies of strains TA98 or TA100 or if it caused a ≥3-fold increase in mean revertant colonies of strains TA1535, TA1537, or TA1538, and there was a dose-response to increasing concentrations of the test material.

3. Quality assurance measures:

A quality assurance statement was signed and dated 12/04/90. This date conforms to the review of the study phases and the draft and the final reports.

IV. RESULTS

1. Analytical Determinations:

Data presented from the analytical determination of the lowest and highest dosing solutions used indicated that the actual concentrations were within 10% of the target concentrations. Similarly, the test material was found to be stable in sterile deionized water for at least 24 hours at room temperature.

2. Preliminary Cytotoxicity Assay: In the dose-rangefinding study, the test material when evaluated at doses ranging from 14 to 14,000 µg/plate, was cytotoxic only at the highest dose (14,000 µg/plate) both in the presence and absence of metabolic activation. Cytotoxicity was evidenced by the reduced number of revertants per plate and, in the absence of S9, the slight thinning of the bacterial background lawn.

3. Mutagenicity Assay: The mutagenicity assay results are presented in Table 3. There was no evidence of a mutagenic response at any dose level and in any strain either in the presence or absence of metabolic activation. The sensitivity of the test system to detect mutagenicity was adequately shown by the response of each tester strain to the appropriate nonactivated and metabolically activated positive control.

V. CONCLUSION: The test material did not cause a positive increase in the number of histidine revertants per plate of any of the tester strains either in the presence or absence of metabolic activation.

There was no evidence that the diethanolamine salt of 2,4-D was mutagenic under the conditons of this assay.

VI. CORE CLASSSIFICATION: Acceptable; this study satisfies the Guideline requirement [84-2(a)] for genotoxic effects category I, Gene Mutation Test.

Table 3. Representative Results of the Salmonella/Microsomal Assay With the Diethanolamine Salt of 2,4-D.

Material	S9	Dose (µg/plate)	Reverents per plate of Bacteril Tester Strain*				
			TA98	TA100	TA1535	TA1537	TA1538
Positive Control							
Sodium azide	-	2.0	--	551 ± 58	484 ± 24	--	--
2-Nitrofluorene	-	1.0	126 ± 19	--	--	--	235 ± 31
ICR-191	-	2.0	--	--	--	123 ± 21	
2-Aminoanthracene	+	2.5	1068 ± 3	1073 ± 78	137 ± 22	149 ± 16	1092 ± 60
Vehicle Control	-	--	17 ± 4	120 ± 12	13 ± 4	6 ± 1	11 ± 2
2,4-D DEA [73.8%]	-	500	22 ± 8	101 ± 8	7 ± 2	6 ± 4	12 ± 3
	-	1000	16 ± 2	120 ± 13	11 ± 2	7 ± 3	18 ± 3
	-	2500	15 ± 6	116 ± 20	13 ± 2	7 ± 2	12 ± 6
	-	5000	17 ± 10	126 ± 8	16 ± 3	8 ± 1	9 ± 3
	-	10,000	17 ± 8	101 ± 11	12 ± 3	4 ± 2	7 ± 2
	-	14,000	13 ± 2	73 ± 9	14 ± 2	5 ± 3	14 ± 3
Vehicle Contol	+	-	29 ± 11	137 ± 12	14 ± 3	11 ± 1	20 ± 7
2,4-D DEA [73.8%]	+	500	30 ± 8	112 ± 5	14 ± 1	6 ± 2	19 ± 2
	+	1000	32 ± 3	137 ± 2	14 ± 5	7 ± 2	17 ± 2
	+	2500	31 ± 2	126 ± 11	10 ± 4	6 ± 2	19 ± 5
	+	5000	32 ± 8	129 ± 13	13 ± 1	6 ± 1	20 ± 6
	+	10,000	28 ± 9	111 ± 6	11 ± 5	5 ± 1	17 ± 5
	+	14,000	20 ± 4	106 ± 5	11 ± 4	7 ± 1	14 ± 4

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PRIMARY REVIEWER: Jess Rowland, M.S, Toxicologist
Section II, Toxicology Branch II

SECONDARY REVIEWER: K. Clark Swentzel, Section Head
Section II, Toxicology Branch II

2/7/92

2/10/92

DATA EVALUATION REPORT

5. RANGE-FINDING STUDY IN RATS

STUDY TYPE: Range-Finding **GUIDELINE:** N/A

Caswell No. 315 K MRID No. 419866-02 HED PROJECT No. 1-2159

TEST MATERIAL: Diethanolamine salt of 2,4-D Acid.

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Springborn Laboratories, Inc. OH

STUDY IDENTIFICATION: SLS 3229.2

TITLE OF REPORT: Range-finding Teratology Study in Rats with Diethanolamine Salt of 2,4-D.

AUTHORS: J.C. Siglin, J.T. Liao and D. E. Rodwell

REPORT DATE: August 31, 1990

SUMMARY: Pregnant Sprague-Dawley rats were given oral administration of 2,4-D DEA [73.09%] at dose 0, 37, 75, 150 or 300 mg/kg/day [equivalent to 0, 27, 55, 110, or 219 mg/kg/day 2,4-D DEA] during gestation days 6-15. Dams were sacrificed on gestation day 20. 2,4-D DEA did not induce maternal or developmental toxicity at 37 or 75 mg/kg/day. Treatment did cause maternal toxicity at 150 and 300 mg/kg/day manifested by mortality, clinical signs, and decreases in body weight gain and food consumption. Minimal developmental toxicity seen at 150 mg/kg/day was characterized by a slight decrease in fetal weight. Developmental toxicity observed at 300 mg/kg/day included: increase in post-implantation loss, decrease in viable fetuses, and reduced fetal weights.

Based on this study the dose levels selected for the main study were 0, 15, 75 and 150 mg/kg/day [equivalent to 0, 11, 55, or 110 mg/kg/day 2,4-D DEA.

CORE CLASSIFICATION: Not applicable; range-finding study.

1. OBJECTIVE

The objective of this range-finding study, was to establish appropriate dose levels of the Diethanolamine salt of 2,4-D for the main study.

2. PROTOCOL

Groups of six mated female Sprague-Dawley Crl:CD BR VAF/Plus rats [80-120 days old] were given oral administrations of 2,4-D DEA (73.09% pure; Batch no.0017) at doses of 0, 37, 75, 150, or 300 mg/kg/day] equivalent to 0, 27, 55, 110, or 219 mg/kg/day 2,4-D DEA] in deionized distilled (10 mL/kg) daily, during days 6 through 15 of gestation. Concentration, homogeneity and stability of the test article/vehicle mixtures were determined prior to the initiation of the study.

Animals were observed for clinical signs of toxicity daily and were weighed on gestation days 0, 6, 9, 12, 16 and 20. Dams in each group were sacrificed on day 20 and postmortem examination included macroscopic examination of internal organs, with emphasis on the uterus, uterine contents, position of each fetus in the uterus, and corpora lutea counts. The uteri (and its contents) of all dams were weighed to determine corrected body weights. The fetuses were sexed, weighed, examined, and discarded.

3. RESULTS

i. Analysis of dosing solution

The mean concentrations found were 100.2%, 99.5%, 94.6% and 97.4% of the nominal concentration for the 37, 75, and 150 and 300 mg/kg dose groups, respectively. The homogeneity analysis showed that the sample solutions were within 5% of the nominal concentrations indicating that the dosing solutions were homogeneous. Stability analysis indicated that 2,4-D DEA was stable in distilled water at room temperature for up to eight days post-preparation.

ii. Maternal Toxicity

- While no mortality occurred at the 37 or 75 mg/kg/day dose groups, one female was found dead on gestation day 12 at 150 mg/kg/day, and one female was sacrificed moribund on gestation day 8 at 300 mg/kg/day.

- Clinical signs of toxicity observed in rats prior to death were decreased activity, tremors, slow breathing and prostration. Less severe clinical signs observed in the surviving dams at 150 and 300 mg/kg/day included reddish vaginal discharge and urine stains. No treatment-related clinical signs were seen at the lower doses.

- No treatment-related effects were noted in body weight gain and food consumptions of dams at 37 or 75 mg/kg/day. Decreases in body weight gain and food consumption were observed at 150 and 300 mg/kg/day. The decrease in maternal weight gain noted at 300 mg/kg/day during gestation days 12-16 and 16-20 was primarily attributable to the reduced gravid uterus weights in these dams.

- Necropsy revealed no treatment-related gross abnormalities and treatment had no adverse effects on the reproductive performances of the dams.

iii. Developmental Toxicity

- No developmental toxicity was observed at 37 or 75 mg/kg/day. Treatment-related effects observed at 150 and 300 mg/kg/day are tabulated below:

PARAMETER	0 mg/kg/day	150 mg/kg/day	300 mg/kg/day
Post implantation loss (%)	7	3	41
Viable fetuses (%)	93	87	59
Gravid uterine weight (g)	76.7±11.12	78.6± 9.98	39.8±28.98
Fetal weight (g)	3.6±0.2	3.2±0.2	2.2±0.2

- No developmental variations or malformations were observed in any fetuses at the tested doses.

4. CONCLUSION

Based on the results of this study [maternal and developmental toxicity at 300 mg/kg/day], dose levels selected for the main study [0, 15, 75 and 150 mg/kg/day] seem appropriate .

5. CORE CLASSIFICATION: Not applicable; study not required by guideline.

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PRIMARY REVIEWER: Jess Rowland, M.S, Toxicologist
Section II, Toxicology Branch II

Jess Rowland 2/10/92

SECONDARY REVIEWER: K. Clark Swentzel, Section Head
Section II, Toxicology Branch II

K. Clark Swentzel 2/10/92

DATA EVALUATION REPORT

6. TERATOLOGY STUDY IN RATS

STUDY TYPE: Teratogenicity/Rat **GUIDELINE:** 83-3(a)

Caswell No. 315 K MRID No. 419209-06 HED PROJECT No. 1-2159

TEST MATERIAL: Diethanolamine salt of 2,4-D

REGISTRANT: PBI Gordon Corporation, Kansas City, MO

TESTING LABORATORY: Springborn Laboratories, Inc. OH

STUDY IDENTIFICATION: SLS 3229.3

TITLE OF REPORT: Teratology Study in Rats with Diethanolamine Salt of 2,4-D.

AUTHORS: J.C. Siglin, M.D. Mercieca and D. E. Rodwell

REPORT DATE: December 12, 1990

SUMMARY: Oral administration of the diethanolamine salt of 2,4-D DEA [73.09%] at doses of 0, 15, 75, or 150 mg/kg/day [equivalent to 0, 11, 55, or 110 mg/kg/day] to mated rats during gestation days 6 through 15 resulted in maternal toxicity manifested by decreases in mean body weight gain and mean food consumption at 75 and 150 mg/kg/day. No maternal toxicity was observed at 15 mg/kg/day. No treatment-related differences were seen in cesarian section parameters between the treated and control groups. The singular fetotoxicity observed at 150 mg/kg/day was a statistically significant reduction in fetal body weight. No treatment-related fetal malformation or variation was observed at 15 mg/kg/day. Treatment-related fetal variations observed at the higher doses included increased incidence of reduced ossification of the skull, and increased incidence of 14th rudimentary ribs at 75 and 150 mg/kg/day groups. Based on the results of this study the following NOELs and LOELs are established for Maternal and Developmental Toxicity:

NOEL: 15 mg/kg/day or 11 mg 2,4-D DEA/kg/day
LOEL: 75 mg/kg/day or 55 mg 2,4-D DEA/kg/day

CORE CLASSIFICATION: Minimum; this study satisfies the requirements for a developmental toxicity study in rats (83-3 a) and is acceptable for regulatory purposes.

1. OBJECTIVE

The objective of this study was to assess the effects of the diethanolamine salt of 2,4-D [2,4-D DEA] on the embryonic and fetal development following oral administration to rats during the period of organogenesis.

2. MATERIALS AND METHODS

a. Test Material

Identity: Diethanolamine salt of 2,4-D
Batch No.: 0017
Purity: 73.09%
Description: Clear, light brown viscous liquid

b. Test Animals

Species/Sex: Female rats
Strain: Sprague-Dawley Crl:CD BR VAF/Plus
Age at Breeding: 84 days
Body Weight on gestation day 0: 228 to 291 g
Identification: Ear tags.
Acclimation: 11 days
Housing: Individually in cages with softwood bedding
Food: Purina Certified Laboratory Chow #5002 ad libitum.
Water: Tap water ad libitum
Environment: Temperature - 72 ± 6°C; Humidity - 30-70%;
Light/Dark- 12 hr.cycle

Group Assignment: 25 mated females were randomly assigned to 1 control group and 3 treatment groups.

c. Mating

Following acclimation, females were housed with sexually mature males (1:1) until mating had been observed (designated day 0 post coitum).

d. Preparation of Dosing Solutions

The test material/vehicle mixtures (w/v) were prepared fresh daily prior to dosing. The vehicle, deionized distilled water, was added to a weighed amount of the test material and the mixture was kept homogenous by constant stirring on a magnetic stirrer for ten minutes prior to daily dispensation.

e. Analysis of the Dosing Solutions

Concentration, homogeneity, and stability analysis of the dosing solutions were determined once during the study.

f. Administration of Test Article

The test article was administered daily orally via gavage at nominal concentrations of 15, 75, or 150 mg/kg/day [equivalent to 0, 11, 55, or 110 mg/kg/day] from day 6 through 15 of gestation. The control group received the vehicle only. All groups received a dosing volume of 10 mL/kg body weight, with a daily adjustment of the individual volume to the actual body weight.

g. Observations

All animals were observed once daily for clinical signs of toxicity including physical or behavioral abnormalities. In addition, during the treatment period, the rats were observed between one-half and two hours following dosing for detection of overt signs of toxicity. Body weights were obtained on days 0, 6, 9, 12, 16 and 20 of gestation. Food consumptions were measured for gestation days 0-6, 6-9, 9-12, 12-16, 6-16, 16-20.

h. Termination

Dams were sacrificed on gestation day 20, and post mortem examination included gross macroscopic examination of all internal organs with emphasis on uterus, uterine contents, position of fetuses in the uterus and the number of corpora lutea. The uteri (and its contents) of all females with live fetuses were weighed and the corrected body weights were calculated. Fetus was weighed, sexed and examined for gross external abnormalities and was prepared by Wilson's slicing technique for visceral examinations and stained with alizarin red S for skeletal examinations.

i. Statistical Analysis

Test Employed	Parameters Analyzed
ANOVA followed by Dunnett's Test	Maternal and fetal data including body weights, food consumption, number of viable fetuses, implantation sites, and corpora lutea
Mann-Whitney U test	Post-implantation loss, dead fetuses, and resorptions
Chi-square test	Fetal sex ratios
Fisher's Exact Test	Fetal malformations and variations.

j. Quality Assurance Measures:

A quality assurance statement was signed and dated 12/18/90. This date conforms to the review of the study phases and the draft and the final reports.

3. RESULTS

a. Analysis of the Dosing Solutions

The mean concentrations found were in the range of 98.5 to 100.3% of the nominal concentration. Homogeneity and stability analyses indicated that 2,4-D DEA was homogenous and stable in aqueous solution when stored for up to eight days at room temperature protected from light.

b. Maternal Mortality

No mortality occurred during the study.

c. Clinical Signs

With the possible exception of increased urine staining and vaginal discharge at 150 mg/kg/day, there were no clinical signs of toxicity that could be directly attributable to treatment.

d. Maternal Body Weight

- No differences in body weight or body weight gain were seen between the vehicle control and 15 mg/kg/day groups.

- At 75 mg/kg/day, a slight, but statistically significant [$p < 0.05$] decrease in mean body weight was seen on gestation day 12 [309 ± 17.9 g vs. 324 ± 23.3 g in vehicle controls]. Body weight gain was significantly [$p < 0.05$] reduced during days 6 - 9 of dosing [8 ± 9.5 g vs.14 ± 6.5 g in vehicle controls], and also during days 6-16 [47 ± 15.8 g vs. 58 ± 16.4 g]. Decreases in corrected mean body weight gain were seen during days 0 - 20 [73 ± 10.8] when compared to vehicle controls [86 ± 17.2].

- At 150 mg/kg/day, body weight gain was significantly [$p < 0.05$] reduced during days 6 - 9 [4 ± 10.2 vs. 14 ± 6.5 g in vehicle controls].

e. Maternal Food Consumption

Except for statistically significant [$p < 0.05$] decreases at 150 mg/kg/day during gestation days 6-9 and 6-16, no other differences in food consumption were noted.

f. Maternal Macroscopical Examination

No treatment-related macroscopical changes were observed in the dams sacrificed at termination.

g. Reproduction Data

There was an incidental statistically significant ($p < 0.05$) reduction in the mean post-implantation loss at 15 and 75 mg/kg/day, 4.4% and 4.9% respectively, when compared to vehicle controls 10.5%. This difference, however, was attributable to a higher than normal mean post-implantation loss in the vehicle control group due to one control female with 100% litter resorption. No other treatment-related differences in cesarean section parameters were seen between the treated and vehicle control groups.

h. Sex Ratios

The sex ratios of fetuses among the treated groups were comparable to that in the vehicle control group.

i. Fetal Weight and Viability

Mean fetal weight was significantly [$p < 0.01$] decreased at 150 mg/kg/day [3.4 ± 0.4] when compared to the vehicle control [3.7 ± 0.3].

j. External Examination

No statistically significant differences were seen in fetal malformations. Although total percentage of litters with malformations was slightly higher at 150 mg/kg/day [13%] compared to vehicle control [4.3%], the types of malformations were generally dissimilar and the incidence of individual malformations remained well within each respective historical control rangee.

k. Visceral Examination (Wilson Technique)

No visceral abnormalities were noted in the 160, 185, 165 or 165 fetuses of the vehicle control, 15, 75, and 150 mg/kg/day groups, respectively.

1. Skeletal Examination

Significant fetal variations observed are tabulated below:

Fetal Variants	Dose Level [mg/kg/day]				
	0	15	75	150	Historical Controls Litter/Total (%)
Number of litters	23	24	21	23	511
Reduced ossification of the skull	5 22%	3 13%	14* 67%	12 52%	17 3% Range: 0-20%
14th rudimentary rib(s)	4 17%	6 25%	9 43%	15* 65%	93 18% Range:3.7- 58.3
7th cervical rib(s)	1 4%	1 4%	2 10%	7* 30%	15 3% Range: 0 - 12%
Bent rib(s)	0 0%	0 0%	6* 29%	4 17%	20 4% Range:0 - 16%

Reduced ossification of the skull: This anomaly is attributed to treatment since the increase is not only statistically significant [p <0.05] at 75 mg/kg/day but also greater than historical controls]. Additionally, the increase at 150 mg/kg/day [52%] while not statistically significant, is also higher than the concurrent control [22%], and outside the of the historical control range [0-20%].

14th rudimentary ribs: A clear treatment-related and nearly a dose-response effect is seen. The increases at 75 mg/kg/day [43%] and at 150 mg/kg/day [65%] are higher than both the concurrent control [17%] and historical controls [18%] with the increase reaching statistical significance [p <0.05] at 150 mg/kg/day.

7th cervical ribs: Although the increases at 75 and 150 mg/kg/day were minimal, it was statistically significant at the high dose.

Bent ribs: The increase was statistically significant [p <0.05] at 75 mg/kg/day [also outside the historical control range], and at 150 mg/kg/day while not statistically significant, the incidence [17%] was also higher than the of the historical controls [4%].

4. DISCUSSION

Oral administration of the diethanolamine salt of 2,4-D at 0, 15, 75, or 150 mg/kg/day to mated rats during days 6 through 15 of gestation resulted in maternal toxicity manifested by decreases in mean body weight, body weight gain, and mean food consumption at the 75 and 150 mg/kg/day groups. No maternal toxicity was observed at the 15 mg/kg dose. No treatment-related differences were seen in cesarian section parameters between the treated and control groups. The slight fetotoxicity observed at 150 mg/kg/day was a statistically significant reduction in fetal body weight.

No statistically significant or treatment-related fetal anomalies were seen at 15 mg/kg/day. Fetal anomalies were seen at the higher doses. The increased incidence of reduced ossification of the skull is attributable to treatment since the increase at 75 mg/kg/day is significantly higher than the concurrent control, and also is out side the historical control range. The incidence of this anomaly at 150 mg/kg/day while not statistically significant is also higher than the concurrent control, and is out side the historical control range. The increased incidence of 14th rudimentary ribs is also attributable to treatment as evidenced by a near dose-response relationship [17%, 25%, 43% and 65% at 0, 15, 75, and 150 mg/kg/day, respectively], with the increase reaching statistical significance at the high dose.. The anomalies of the 7th cervical ribs and the bent ribs is also attributable to treatment due to statistically significant increases at 150 and 75 mg/kg/day doses, respectively, [also outside the historical control range]; however, the biological significance of these variants are not known due to the lack of a dose-response.

The report concluded that "the biological significance of these changes [fetal variations] was not clear since the increases occurred only at maternally toxic levels". Based on this observation, the authors established a NOEL of 150 mg/kg/day for developmental toxicity.

This reviewer does not agree with the author's conclusion that the NOEL for developmental toxicity is 150 mg/kg/day. It is clear that two of the fetal anomalies, namely the increased incidence of reduced ossification of the skull and the 14th rudimentary ribs, seen at the 75 and 150 mg/kg/day are due to the adverse developmental effect of the test material. In addition, even if these effects can be associated with manifestations of maternal toxicity, such effects are still toxic manifestations and such are generally considered a reasonable basis for Agency regulation and/or risk assessment. Consequently, a NOEL of 15 mg/kg/day is established for developmental toxicity.

5. CONCLUSION

2,4-D DEA is a developmental toxin when administered orally to pregnant rats during the period of organogenesis. Based on the results of this study the following NOELs and LOELs are established:

Maternal Toxicity

NOEL: 15 mg/kg/day or 11 mg 2,4-D DEA/kg/day
OEL: 75 mg/kg/day or 55 mg 2,4-D DEA/kg/day

Developmental Toxicity:

NOEL: 15 mg/kg/day or 11 mg 2,4-D DEA/kg/day

LOEL: 75 mg/kg/day or 55 mg 2,4-D DEA/kg/day

6. **CORE CLASSIFICATION: Minimum;** this study satisfies the requirements for a developmental toxicity study in rats (83-3 a) and is acceptable for regulatory purposes.

END